PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF GINGER (*Zingiber officinale Rosc.*) FROM SPROUTED RHIZOME BUD EXPLANTS

MSC. THESIS

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Jimma University

PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF GINGER (*Zingiber officinale Rosc.*) FROM SPROUTED RHIZOME BUD EXPLANTS

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By

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Jimma University

DEDICATION

I dedicated this thesis to the memory of my sister Tirudel Chekole.

STATEMENT OF THE AUTHOR

I declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for M.SC. Degree at Jimma University and is deposited at the University Library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

The author was born on July 19, 1991 G.C in Mender Maryam Kebele, Libokemkem Woreda, South Gonder, Amahara Regional State, Ethiopia. She had attended her primary school education at Mikihal Debir Primary and Junior School from 1989 to 1996 E.C. She attended her secondary and preparatory education at Maksegnit Secondary School from 1997 E.C to 2000 E.C. She joined Debire Birhan University in 2001 E.C. and completed her undergraduate studies with BSc. Degree in plant science in 2003 E.C. She was employed by Ministry of Education as a graduate assistant in 2004 E.C. at Wolaita Soddo University. After she served for one year in Wolaita Soddo University, she joined the School of Graduate Studies of Jimma University to pursue her M.Sc study in Plant Biotechnology in September 2012 G.C.

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LIST OF ABBREVIATIONS

2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6- Benzyl amino purine
FAO	Food and Agriculture Organization
GA3	Gibberellins Acid
IAA	Indole acetic acid
IBA	Indole-3- butyric acid
Kin	Kinetin
MoARD	Ministry of Agriculture and Rural Development
MS	Murashige and Skoog
NAA	α -naphthalene acetic acid
PGR	Plant Growth Regulator
TDZ	Thidiazuron
UEPB	Uganda Export Promotion Board

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ABSTRACT

Although ginger is an important crop in Ethiopia, its production is limited by different factors like inefficient propagation method and disease transmission. Therefore, this study was initiated to develop a protocol for in vitro propagation of ginger from sprouted rhizome buds. Sprouted rhizome buds were cultured as whole and divided into half longitudinally in MS medium supplemented with different concentration of TDZ (0,0.1,0.3,0.&0.7) and BAP (0,1,2, 3& 4) mg/l to evaluate their effect on shoot multiplication. This particular experiment was laid in 2 x 5x5 factorial arrangement for types of explants, concentrations of BAP and concentrations of TDZ, respectively. Micro shoots were then transferred into 1/2MS medium supplemented with different combination of NAA (0, 0.5, 1, 1.5 & 2.0) and IBA (0, 0.5, 1, 1.5& 2.0) mg/l for root induction. Both experiments were laid out in completely randomized design (CRD) with three replication and three explants per jar. The cultures were kept at a temperature of 25 ± 2°C and light intensity of 2,000-µmol/m^2/ s^2 produced from cool white fluorescent tubes for 16 h photoperiod. Finally well rooted shoots were transferred into greenhouse for acclimatization. Data on number of day for shoot initiation, shoot number, shoot length and leaf number (for shoot multiplication) and number of days for root initiation, root number and root length (for rooting) were collected. The data was analyzed using SAS software (version 9.2). Statistical analysis revealed that there was significant difference among all treatments in both shoot multiplication and rooting experiment. Maximum numbers of shoots per explant (5.33±0.58) and (7.00±0.00) were obtained from MS medium containing 1.0 mg/l BAP combined with 0.5mg/l TDZ for whole and section bud explants respectively. The lowest shoot (2.33±0.58) number was observed from higher combination of the two hormones followed by MS medium with no hormone added in both explants. The highest number of induced roots per shoot (10.00±0.00) was obtained at 1.5 mg/l IBA combined with 0.5mg/l NAA. Among the rooted plantlets used for acclimatization, 80% of them survived. According to the result obtained in this study, MS medium supplemented with 1.0 mg/l BAP combined with 0.5mg/l TDZ for shoot multiplication and sectioned rhizome bud explants and 1/2MS medium supplemented with 1.5mg/l IBA combined with 0.5mg/l NAA are recommended for in vitro propagation of ginger from sprouted rhizome bud explants. However, further optimization of this protocol may be required for mass propagation of ginger as this study is limited to single of cultivar.

Key words/Phrases*:* BAP, TDZ, NAA, IBA, Rooting, Shoot Multiplication, Growth Regulators

1. INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is an herbaceous perennial plant belonging to the family *Zingiberaceae*. It originated in South East Asia and introduced to many parts of the world; and has been cultivated for thousands of years as spice and medicinal plant in India and China (Shulka and Singh, 2007). Ginger is cultivated in several regions of the world such as India, China, Japan, Indonesia, Australia, Nigeria and West Indies islands. Among them India and China are the dominant suppliers to the world market. The total production of ginger in the world is 1,683,000 tons with the total area coverage of 310,430 ha (FAO, 2010).
The cultivation of ginger was started in Ethiopia during 13th century when Arabs introduced it from India to East Africa (Jansen, 1981). It is cultivated in South, South western and Northwestern parts of Ethiopia as a cash crop, and is among the important spices used in every Ethiopian kitchen for the preparation of pepper powder, stew, bread, etc.

Ginger production for the extraction of oleoresins essential oils, as well as the direct use of rhizomes for culinary purposes is increasing worldwide (FAO, 2008). It is a crop extensively grown across the world for its pungent aromatic underground stem or rhizome which makes it an important export commodity in world trade (Ajibade &Dauda, 2005).

It has also some use in traditional medicine for the treatment of flu and stomach ache. Currently, it has become an important cash crop for farmers in southern and south-western parts of Ethiopia. It is the second most widely cultivated spice in Ethiopia, next to chilies. In Ethiopia, ginger is grown in an area of 45,164ha with production of 15.87 tons per hectare (MoARD, 2007). It is limited mostly in the wetter regions of Southern Nations, Nationalities and Peoples Regional State (SNNPRS) and some parts of western Oromia. There are also cultivations (in small quantities) in Gojam and Gonder for home consumption (Nigist and Sebsebe, 2009).

Ginger rhizome is typically consumed as a fresh paste, dried powder, slices preserved in syrup, candy (crystallized ginger), as a beverage or as flavoring agent. In many countries, especially in India and China, fresh ginger is used to prepare vegetable and meat dishes and used in preparation of various foods for seasoning, flavoring and imparting aroma all over the world (Shukla and Singh, 2007).

Both fresh and dried ginger rhizomes are used worldwide as a spice and ginger extracts are used extensively in the food, beverage, and confectionary industries in the production of products such as marmalade, pickles, chutney, ginger beer, ginger wine, liquors, biscuits, and other bakery products (Parashuram, 2009). Ginger is a multi-functional crop and its rhizome quality affects the economic return of the growers, its establishment & growth and yield of the crop.

Ginger has been affected by biotic and a biotic factors. Among the biotic factors various fungal and bacterial diseases cause crop losses. These include soft rot (*Pythium sp, Rhizoctonia sp)*, dry rot (*Fusarium oxysporum*) and bacterial wilt (*Ralstonia solanacearum*). Some of these diseases, particularly bacterial wilt once introduced into cultivated fields, it is very difficult to eradicate (Dohroo, 2005. Propagation by conventional means is hampered due to slow propagation rate and the risk of disease transmition through rhizomes. Therefore, *in vitro* techniques are considered the best alternatives method that may supply a clean and large number of planting materials for commercial planting (Hamirah *et al.* 2007). Millions of disease free plantlets can be produced within 1-2 years from a single explant (Sahavacharin and Thaveecha 1997).

Suitable explant with optimum size is required for successful initiation of cultures. Very small explants do not survive well in cultures whereas large explants may be difficult to decontaminate effectively or are less easily manipulated (George *et al.* 2009). The different types of explants used in micro propagation of ginger and other related species are meristem, axilliary buds, shoot tips and aerial pseudo stems. However, rhizome buds and shoot tips are commonly used as explants and they are the responsive explants for micro propagation of pathogen free propagules on a large scale (Lincy and Sasikumar, 2010).

Behera *et al.(*2010) could produce plantlets easily using sprouting bud explants of ginger and turmeric. De Almeida *et al.,* (2002) who stated that longitudinal sectioning of the shoots used as explants proved to be a very good technique to improve the multiplication of shoots in paean apple. They reported that the average number of shoots per explants of the sectioned explant was high (613.7) compared to only 69.1shoots/explant when the whole shoot was used as explant. Sectioning in meristematic regions can induce cell division due to the high mitotic ability of these cells (George, 1993).

The stimulation of cell division, caused by the sectioning of the shoot, probably contributed for the differentiation of a higher number of adventitious buds and consequently a higher number of shoots produced per explants.

TDZ has been shown to exhibit stronger effect than other conventionally used cytokinins (Iram & Anis, 2007) for shoot multiplication. Prathanturarug *et al.*(2003) obtained18.22 shoots/explants after 12 weeks by using 18.17 µM TDZ. Dipti *et al.* (2005 who reported that the highest number of multiple shoots in media supplemented with 2 mg/l BAP in shoot tip and 3 mg/l BAP in rhizome bud in turmeric, proved its superiority over KIN producing more number of multiple shoots. Explant type and plant growth regulator, concentration and combinations may have different result. Several researchers have used different explant types for *in vitro* propagation of ginger but there is no information on such work done in Ethiopia. Therefore the present study was conducted to develop a protocol for *in vitro* propagation of ginger using different explant types and types of hormone and their combinations.

General objective

- To develop *in vitro* propagation protocol for rhizome explants using ginger (Zingiber officinale Rosc.).

Specific objectives

- To determine the optimum combined concentrations of BAP and TDZ for shoot multiplication.
- To determine the optimum combined concentrations of IBA and NAA for rooting
- To identify a suitable explant type for *in vitro* multiplication.

2. LITERATURE REVIEW

2.1. Origin and Production of Ginger (*Zingiber Officinale)*

Ginger originated in South-East Asia, probably in India. It is cultivated in several parts of the world, the most important producing regions being India, China, Nigeria, Sierra Leone, Indonesia, Bangladesh, Australia, Fiji, Jamaica and Nepal. Among them, India and China are the dominant suppliers to the world market (Peter 2001). The major ginger growing area in Ethiopia includes wetter regions at altitude bellow 2000 m in Kefa, Illubabur, Gamo Gofa, Sidama, and Wellega mostly in garden and around homesteads. Large scale production and marketing of ginger are also reported from Illubabur, Wolaita, Kembata-Tambaro, etc. Ginger is the cash crop for the Gumuz people in Benshangul Gumz region. Currently, it has become an important cash crop for farmers in southern and south-western parts of Ethiopia. There are also cultivations (though in small quantities compared to the wet parts of the country) in Gojam and Goner to cover home consumption. The production of this spice has been expanding in most parts of the country, as it can be grown under varied climaties that do not have frost problem. Ginger thrives well in areas with altitudes from sea level to 1500 m, mean annual temperature of 20 - 32 ^oC and with total rainfall greater than 1200 mm. The ideal soil type for the production of ginger is a well-drained, fertile and friable soil and with enough humus, neutral pH. Coffee soils or forest soils of south and south-western Ethiopia, having comparable varieties of soil with above ones, especially around Tepi and Bebeka are found suitable for ginger production (Nigist and Sebsebe 2009).

2.2. Botany of Ginger

Ginger belongs to the family Zingiberaceae of the natural order Scitaminae. The family Zingiberaceae consists of 47 genera and about 1400 species. Among these, 22 genera and 178 species is endemic to India. The genus *Zingiber* consists of 80-90 species. Among these *Z. zerumbet* and *Z. cassumunar* are medicinal species and *Z. officinale* is the cultivated ginger (Mohanty and Panda, 1994). Ginger is probably a sterile hybrid between two distant species, but survived because of the successful vegetative mode of propagation (Sato, 1960).

Ginger is an herbaceous perennial having underground branched rhizome with small scales. The inner core of the rhizome is pale yellow to bluish tinge while the outer is light yellow. Adventitious roots and storage roots arise from among the nodes of these scales. The ancillary buds shoot up as leafy stem known as pseudo stem, which die out annually but the plant continues to live through its rhizome. Leaves are sheathing arranged alternatively, linear lanceolate, gradually acuminate and glabrous. Flowers are borne on a spike produced in a peduncle different from the aerial leafy stem, arising directly from the rhizome. The spike is condensed, oblong and cylindric with numerous imbricate bracts, persistent and each carrying two flowers. Flowers are many, trimerous bisexual, irregular, epigenous, yellow in colour with dark purplish spots. Outer periapt is cylindric, shortly three lobed, inner periapt tube is cylindric, lobes are lanceolate., one stamen perfect, two combined into a petaliferous leaf, the labellum. Androecium consists of stamens of which the outer 3 are reduced to stamenoids. The inner lateral stamens are united and showy to form a deep purple coloured labellum. The posterior stamen of the inner whorl is the only fertile stamen, which is enclosed by the labellum. The filament is flat and short with two prominent anther lobes. The style passes through the grove formed by the anther lobes and ends in a capitate stigma. Anther cells are contiguous, produced into a long beak. Ovary is inferior, three carpelled, three celled. Ovules are many on axial placentation. Style is long, delicate, lying in a groove in the stamen. Stigma is small and sub-globose. Fruit is very rarely produced, which is an oblong capsule (Ravindran and Babu 2005). But *Zingiber officinale* is not known to set seeds.

The floral biology of ginger was studied at Kasaragod(AICSCIP, 1975). The flowers open between 14.30 and 16.30 hours and anthesis take place simultaneously. It takes about 20-25 days from the flower bud initiation to full boom. In an inflorescence blooming takes place in 23-28 days in an acro-petal sucession. Anthesis takes place between 1.30 P.M to 3.30 P.M. Anther dehiscence almost coincided with the flower opening or followed it immediately. Stigma is receptive at the time of anther dehiscence. Flowers were hermaphrodite with pin and thrum type incompatibility, and dehisced pollen grains did not reach the stigma head. No seed set was obtained by selfing or crossing. The somatic chromosome number of ginger is 2n = 22 in all species except in *Z. mioga* (2n=55).

2.3. Use of Ginger

Both fresh and dried ginger rhizomes are used worldwide as a spice, and ginger extracts are used extensively in the food, beverage, and confectionary industries in the production of products such as marmalade, pickles, chutney, ginger beer, ginger wine, liquors, biscuits, and other bakery products (Mishra, 2009).In Ethiopia it is among the important spices used in every kitchen to flavor stew, tea, bread and local alcoholic drinks (Nigist and Berhanu 1995).

Ginger is also widely used in both traditional and contemporary natural medicine. It has been used medicinally in India since ancient times. Ginger is included in the British, European, Chinese, and Japanese pharmacopoeias, as well as in many other national pharmacopoeias, and the World Health Organization has published a monograph for *Rhizoma zingiberis*. The medicinal uses of ginger are diverse and include for indigestion, stomachache, malaria and fevers. It is chiefly used to cure diseases due to morbidity of Kapha and Vata. Ginger with lime juice and rock salt increases appetite and stimulates the secretion of gastric juices. It is said to be used for abdominal pain, anorexia, arthritis, atonic dyspepsia, bleeding, cancer, chest congestion, chicken pox, cholera, chronic bronchitis, cold extremities, colic, colitis, common cold, cough, cystic fibrosis, diarrhoea, difficulty in breathing, dropsy, flatulent, disorders of gallbladder, hyperacidity, hypercholesterolemia, hyperglycemia, morning sickness, prevention of motion sickness, nausea, rheumatism, sore throat, throat ache, stomach ache and vomiting in pregnancy Mishra(2009).

2.4. In Vitro Propagation of Ginger

Clonal propagation through tissue culture, popularly called, micro propagation, can be achieved in a short time and space. Thus, it is possible to produce plants in large numbers from a single individual. Micro propagation technology owns unique distinction as the quick and easy method of deriving plants with identical genetic constitution (Hussey, 1986).

It has a significant impact on plant breeding, horticulture, and medicine. This technique is an alternative method of propagation as there is an increase in the propagation rate of plants, availability of plants throughout the year, protection of plants against pests and pathogens

under controlled conditions and the availability of uniform clones and uniform production of secondary metabolites (Bajaj *et al.,* 1988).

2.4.1. Media compositions

Growth and morphogenesis of plant tissues *in vitro* is largely governed by the composition of the culture media. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species and the type of tissue under consideration (George and Klerk, 2008). Considerable progress had been made in the past few decades on the development of media formulations for growing plant cells, tissues, and/or organs aseptically *in vitro*. A significant contribution to formulation of a defined growth medium suitable for a wide range of applications was made by Murashige and Skoog (1962), in their work to adapt tobacco callus cultures for use as a hormone bioassay system; They evaluated different media constituents to achieve optimal callus growth.

Several media formulations are commonly used for cell and tissue culture works for the regeneration of specific plant species from different explant types. Among others, the most frequently used media formulations in tissue culture include those described by White, Murashige and Skoog, Schenk and Hildebrandt, Nitsch and Nitsch, and Lloyd and McCown (Phytotechnology Laboratories, INC, 2003).Culture media used for *in vitro* cultivation of plant cells are composed of some or all of the following basic components: Macro elements (or macronutrients), microelements (or micronutrients), organic supplements, when deemed necessary, a carbon source, gelling agents, plant growth regulators, and sometimes even antibiotics.

2.4.1.1. Macronutrients

Novak *et al*. (1983) studied the macronutrients provide the six major elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) required for plant cell or tissue growth. All are essential for plant cell and tissue growth. Culture media should contain at least 25 $mmoll^{-1}$ nitrates and potassium.

However, considerably better results are obtained if both nitrates and ammonium (2-20 mmoll-1) or any other reduced nitrogen source contributes the source for nitrogen in media. Hall (1999) pointed out that in case only ammonium is used, there is a need to add one or more tricarboxylic acid (TCA) cycle acids (e.g., citrate, succinate, or malate) so that any deleterious effect due to ammonium concentrations in excess of 8 $mmoll^{-1}$ in the medium is diluted. When nitrate and ammonia ions are present together in the culture medium, the latter are used more rapidly. Other major elements, Ca, P, S, Mg; at concentrations in the range of 1-3 $mmoll^{-1}$, appear adequate provided other requirements are satisfied.

2.4.1.2. Micronutrients

These elements are required in trace amounts for plant growth and development. Manganese(Mn), iodine(I), copper(Cu), cobalt(Co), boron(B), molybdenum(Mo), iron(Fe) and zinc(Zn) are regarded as microelements, although other elements like aluminium and nickel are frequently found in some formulations.

Chelated forms of iron and zinc are commonly used in preparing culture media. Iron citrate and tartrate may be used in culture media, but these compounds are difficult to dissolve and frequently precipitate after media are prepared. Murashige and Skoog (1962) used an ethylene diamineteracetic acid (EDTA) iron chelate to bypass this problem.

Several researchers recommended that cobalt (Co) and iron (Fe) may also be added to certain media, but strict cell growth requirements for these elements have not been established. Sodium (Na) and chlorine (Cl) are also used in some media but are not essential for cell growth (phytotechnology laboratories, INC, 2003).

2.4.1.3. Organic supplements

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins (including some substances that are not strictly animal vitamins), amino acids and certain undefined supplements. The amount of

these substances required for successful culture varies with the species and genotype, and is probably a reflection of the synthetic capacity of the explant (George *et al.,* 2008).

Vitamins: Plants synthesize vitamins endogenously and these are used as catalysts in various metabolic processes. According to Andrew and Marin (2005), the vitamins most frequently used in cell and tissue culture media include thiamine (B1), nicotinic acid (B3), pyridoxine (B6), calcium pentothenate (B5), and my oinositol. The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture.

Amino acids: Cultured tissues are normally capable of synthesizing the amino acids necessary for various metabolic processes. In spite of this, the addition of amino acids to media is important for stimulating cell growth in protoplast cultures and for establishing cell lines. Amino acids provide plant cells with an immediately available source of nitrogen, and uptake can be much more rapid than that of inorganic nitrogen in the same medium (Thom *et al.,* 1981).

Activated charcoal: The addition of activated charcoal (AC) to culture media is reported to stimulate growth and differentiation in orchids, carrot, ivy and tomato. Paradoxically, its effect on tobacco, soybean and Camellia has proved inhibitory (Owen *et al.,* 1995). It also helps to reduce toxicity by removing toxic compounds (e.g. phenols) produced during the culture and permits unhindered cell growth.

Other organic supplements: Culture media are often supplemented with a variety of organic extracts which have constituents of an undefined nature. These include protein (casein) hydrolysates, coconut milk, yeast and malt extracts, ground banana, orange juice, and tomato juice. Yeast extract has been shown to have some unusual properties which may relate to its amino acid content (Ayabe *et al.,* 1988). It also stimulated furomocoumarin production in *Glehnia littoralis* cell suspensions (Kitamura *et al*., 1998).

2.4.1.4. Carbon and Energy source

The most preferred carbon source in plant tissue culture is sucrose. Glucose supports equally good growth while fructose is less efficient. Sucrose, while autoclaving the medium, is converted into glucose and fructose. In the process, first glucose is used and then fructose. Other carbohydrates, such as lactose, galactose, rafinose, maltose, cellobiose, melibiose, and

trehalose, generally yield inferior results. Autotrophic cells are capable of fully supplying their own carbohydrate needs by carbon dioxide assimilation during photosynthesis. Plant cells and tissues in the culture medium lack autotrophic ability and therefore need external carbon for energy (Larosa *et al.,* 1981).

2.4.1.5. Plant growth regulators (PGRs)

Some chemicals occurring naturally within plant tissues, i.e. endogenously, have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as plant hormones or plant growth substances.

Five broad classes of growth regulators, namely auxins, cytokinins, gibberellins, abscisic acid, and ethylene, are considered important in tissue culture, as the growth, differentiation and/or organogenesis of tissues become feasible only with the addition of one or more of these classes of chemicals to the culture medium. Skoog and Miller (1957) were the first to report the critical effects of the ratio of auxins to cytokinin in determining the type and extent of regeneration in plant cell culture. In general, the ratio of hormones required for root or shoot induction varies considerably with the tissue, which seems directly correlated to the quantum of hormones synthesized at endogenous levels within the cells.

Auxins: These are very widely used in plant tissue culture and usually form an integral part of nutrient media. In many cases, the auxins commonly used in tissue culture media are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) (Engvild, 1985). The most commonly detected natural auxin is IAA (indole-3-acetic acid) but endogenous occurrences of 4-chloro-IAA and of indole-3-butyric acid (IBA) have also been demonstrated. Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis. At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division they are involved in the formation of meristems giving rise to either unorganized tissue, or defined

organs. In organized tissues, auxins are involved in the establishment and maintenance of polarity and in whole plants their most marked effect is the maintenance of apical dominance and mediation of tropisms (Davies, 2004).

IAA and to some extent also IBA are heat labile and decompose during autoclaving. IAA is also unstable in culture media at room temperature. In the dark, there can be more than a ten-fold decrease in concentration over a four-week period in the absence of inocula (Nissen and Sutter, 1988). The rate of decrease of IAA is even more rapid in the light and is accelerated by the presence of MS salts.

Cytokinins: Added to shoot culture media, these compounds overcome apical dominance and enhance the release of lateral buds that otherwise are set dormant. Except in research works, the natural cytokinins 2-iP and Zeatin are not commonly used in commercial laboratories, due to their associated high cost. Fortunately, several chemical analogues of natural cytokinins apart from kinetin have been identified that are found to be highly effective substitutes of these naturally occurring cytokinins (Kaminek *et al.,* 1987). Cytokinins are by large adenine derivatives, are mainly concerned with cell division, modification of apical dominance, and shoot differentiation in the tissue culture. Cytokinins commonly used in culture media include BAP (6-benzyloaminopurine), 2iP (6-dimethylaminopurine), kinetin (N-2-furanylmethyl-1H-purine-6-amine),Zeatin(6-4-hydroxy-3-methyl-trans-2-butenylaminopurine)andTDZ (thiazuron-N-phenyl-N-1,2,3thiadiazol-5ylurea).

The ratio of auxins to cytokinins in a particular culture medium is extremely important with respect to the nature of upcoming morphogenesis in the culture system. Accordingly, when one desires to ensure for embryogenesis, callus initiation, and/or root initiation, it is essential to ensure to maximize the ratio of auxins to cytokinin, while the reverse leads to axillary and shoot proliferation (Iwamura *et al*., 1980).

Gibberellins: These growth compounds are occasionally used in plant tissue culture. In some species, these substances are required to enhance and in others to inhibit growth. GA_3 is the most common chemical of the gibberellin group, which are over 20 in number. The chemical

promotes the growth of cell cultures at low density, enhances callus growth and induces dwarf or stunted plantlets that will be elongated at subsequent stages.

Abscisic Acid (ABA): It has long been known that dehydration of plant tissue leads to increased biosynthesis of ABA However; it is now well established that a number of other environmental factors, including low and high temperatures can also produce the same effect (Kefu *et al*., 1991). In the culture medium, ABA either stimulates or inhibits callus growth depending on the species under consideration. It is used in plant tissue culture to promote distinct developmental pathways, such as somatic embryogenesis, but it inhibits cell division.

Ethylene: Ethylene is associated with controlling fruit ripening in climacteric fruits, and its use in plant tissue culture is not widespread. Some plant cell cultures produce ethylene, which, when left to build up sufficiently, can inhibit the growth and development of the culture. Ethylene does seem to stimulate callus growth of some plants *in vitro*. For example, on a hormone-free medium, *Ginkgo biloba* embryos germinated when placed on medium within tubes sealed with cotton plugs, but gave rise to callus if the tubes were covered with Parafilm (Webb *et al*., 1986).

2.4.1.6. Gelling agents

Gelling or solidifying agents are commonly used for preparing semisolid or solid tissue culture media. In static liquid cultures the tissue or cells become submerged and die due to lack of oxygen, thus calling upon such gels that would provide a support to the growing tissues under static conditions. The most common of these include agar-agar, as well as others that are commonly known as **gelatin,** alginate, phytagel and gerlite, etc.

Agar: A polysaccharide obtained from seaweed, has several advantages over other gelling agents. First, agar gels do not react with media constituents. Secondly, they are not digested by plant enzymes and remain stable at all feasible incubation temperatures. Normally, 0.5 to 1% agar is used in the medium to form a firm gel at the pH typical of plant cell culture media (Beruto *et al.,* 1995). They also contain phenolic substances and less pure grades may contain long chain fatty acids, inhibitory to the growth of some bacteria. As agar can be the most expensive component of plant media, there is interest in minimizing its concentration.

Concentrations of agar can be considered inadequate if they do not support explants or lead to hyperhydricity. Hyperhydricity decreases as the agar concentration is raised but there may be an accompanying reduction in the rate of growth (Scherer et al., 1988).

Gelatin: At high concentrations (10%) has been tried as a gelling agent but has limited use because it melts at low temperature (25^0 C). Other compounds successfully tested include biogel (polyacrylamide pellets) alginate, phytagel and Gelrite. The advantage of working with synthetic gelling compounds is that they form clear gels at relatively low concentrations (1.25 - $2.5g1^{-1}$) and are valuable aids for detecting contamination that may develop during the span of cultures. Whether explants grow better on agar or other supporting agents depends on the specific tissues treated and/or the species under consideration (Banik *et al*., 2000).

2.4.2. Type of explants

The different types of explants used in micro propagation of ginger and other related species are meristem, axilliary buds, shoot tips and aerial pseudo stems. However, rhizome buds and shoot tips are commonly used as explants and they are the responsive explants for micro propagation of pathogen free propagules on a large scale (Lincy and Sasikumar, 2010). Behera *et aI.,(* 201 0) were able to produce plantlets easily using sprouting bud explants of ginger and turmeric. Leafy aerial pseudo stems and crown segments of ginger have been also successfully cultured under aseptic conditions (Ikeda and Tanabe, 1989) and meristem culture is very useful for the elimination of viruses from infected plant materials. Vertical sectioning of the bud explant into two haves may be able to double the number of explants than using the whole bud to increase the production (Hamirah *et al.,*2007). It has been reported on micro propagation of pineapple in which the shoot multiplication rate improves remarkably (De Almeida *et al.,* 2002).

2.4.3. Shoot Multiplication

Rahman *et al*.(2004) reported that best shoot proliferation from rhizome bud explants in MS + 2.0 mg/l BAP and gave 6.2 average lengths of shoots per culture. Panda*et al*. (2007) developed *in vitro* propagation protocol for *Curcuma longa* that gave 7.6 shoots by using medium with 3.0 mg/l BAP. Naz *et al. (2009)* reported that the higher concentration of BAP decreases the shoot multiplication rate in turmeric among BAP (1-6 mg /l) tested. Kambaska and Santilata (2009) also indicated that BAP (2.0 mg /l)+NAA (0.5 mg /l) resulted in best response for shoot multiplication (7.5 shoots per rhizome bud) but higher concentration of BAP (2.5-3.0 mg /l) with NAA (0.5 mg /l) exhibits callus formation with fewer number of shoots. These results contrast with BAP at highest dosage is more stimulatory to shoot growth than the lowest dosage in the culture medium (Zuraida *et al.,* 2011). TDZ at 0.5 mg /l induces higher number (8) of shoots among the cytokinins even at lower concentration (Hamirah *al.,* 2007) while Prathanturarug *et al*.(2003),obtained,18.22 shoots/explant after 12 weeks by using 18.17 µM TDZ.

2.4.4. Rooting

Dipti *et al.* (2005) studied *in vitro* multiplication and rooting of shoots in turmeric found the maximum rooting to multiple shoots were observed on half strength MS medium with 0.5 mg/l NAA.Meenakshi *et al.* (2001) who observed that the highest rooting was stimulated by sub- culturing the proliferated shoots on half strength MS medium with 0.3 mg/l NAA during micro propagation of turmeric. Bhagyalakshmi and Singh (1988) stated that IBA is more effective when compared to NAA for root formation in meristem culture. In contrary, NAA was more effective than IBA in induction of rooting (Kambaska and Santilata, 2009). Rooting is best on 0.5 mg /l IBA but rooting of micro rhizomes does not take place in the absence of IBA (Sit and Tiwari, 1998).According to Sharma (2006), IAA and IBA are usually used for easier-to-root herbaceous plants and NAA for more recalcitrant woody plants, and they mentioned that the efficacy of different auxins also depends on the explants type and exposure to light.

2.4.5. Acclimatization

The greenhouse and field conditions are characterized by very high light intensity and low humidity but environments in culture containers are low light intensity and very high humidity (Desjardins *et al.,* 1987). Ali *et al.* (2004) successfully transferred turmeric plantlets to the greenhouse in pots containing soil with equal amount of sand + clay + compost. Plants were successfully established in field with 100 per cent survival rate. Salvi *et al.*(2000) observed complete plants of turmeric were transferred to sterilized soil in paper cups for 3 to 4 weeks and then to the field, where 95 per cent of plants survived to maturity. *In vitro* rooted ginger plants were transplanted into humus soil: kitchen garden soil (3:1) under 24 to 28, 70 to 80 per cent relative humidity in which more than 90 per cent survival rate was recorded. Samsudeen *et al.* (2004) were found that 85 per cent success when plant transplanted in potting mixture of garden soil, sand and vermiculite in equal proportions and kept in humid chamber initially for 22 to 30 days.

3. MATERIALS AND METHODS

3.1. Plant Material

The cultivar used for this study is Boziab (37/79). This cultivar was selected based on its high yield, low fiber content, high oil content, and less oil loss when dry. The cultivar was obtained from Areka Agricultural Research Center (AARC), Southern Agricultural Research Institute. The experiment was conducted in tissue culture laboratory of Areka Agricultural Research Center.

3.2. Media Preparation

MS basal medium (Murashige and Skoog, 1962) was used for shoot multiplication and 1/2MS medium was used for rooting. Sucrose was used as a carbon source. Stock solutions of macronutrients, micronutrients, growth regulators and vitamins were prepared separately and stored at 4℃ for immediate use. Then MS basal media was prepared by mixing the appropriate volumes of stock solutions and finally different concentrations and combinations of growth regulators were added accordingly. After dissolving all the components, the pH was adjusted to 5.8 using 1N NaOH and 1N HCl and then 0.8% agar was added. After melting the prepared media by using hot plate magnetic stirrer, 40 ml medium was dispensed into each culture jar. The culture jars were sealed with paraffin and autoclaved at 121°C and 15psi for 15 minutes. The culture media was then stored at room temperature until used.

3.3. Surface Sterilization and Preparation of Explants

Selected fresh rhizomes were washed with common liquid soap and retained in a moist bed laid with absorbent cotton in store house. The rhizomes were watered regularly using distilled water for 30 days to initiate sprouting. Subsequently, sprouted buds were washed once with sterile distilled water and used as explants. Sterilized explants were then dissected to remove one layer of leaf sheaths under aseptic condition. Then, the excised explants were immersed again in 70% ethanol for 5 minute and 50% local bleach (berekna) for 30 minute and washed 5 times with sterilized distilled water. About 1 to 1.5 cm long explants were cultured as whole bud and bud sectioned longitudinally into half onto the culture medium which contain

antibiotics (streptomicine and gentamicine 1mg/l and tetracycline at 15mg/l) before agar was added.

3.4. Culturing and Culture Condition

All cultures were kept under 16 h photoperiod at a photosynthetic flux of 2,000-µmol/m^2/s ^{2} provided by cool fluorescent lamps and maintained at 25 ± 2°C.And shoot initiation was done in the growth room.

3.5. Experimental Design and Treatments

In this study two different experiments were carried out using completely randomized design (CRD) with three replications and three explants per jar. The explants were randomly cultured with treatment combinations. Growth regulator free media was used as a control.

3.5.1. Experiment 1: The combined effect of BAP, TDZ and explants type on shoot multiplication

Rhizome buds were sprouted in store house laid with in a moist bed absorbent cotton divided in to two half and as whole cultured on MS media supplemented with five different concentrations of BAP (0, 1, 2, 3, 4, mg/l) and (0,0.1,0.3,0.5,0.7mg/l) TDZ. This particular experiment was laid in a 2x5x5 factorial arrangement in CRD, for types of explants, concentrations of BAP and concentrations of TDZ, respectively. Data were collected under this experiment number of days shoot initiation after sub culture, shoot number, shoot length and leaf number.

3.5.2. Experiment 2: The combined effect of IBA and NAA on rooting of micro-shoots

In this experiment ½ MS basal medium with different concentrations and combinations of IBA and NAA were investigated for rooting of micro shoots. Accordingly, five concentrations of IBA (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) and NAA (0.0, 0.5, 1.0, 1.5, 2 mg/l) with 5x5 treatment combinations were used. Data was collected under this experiment number of days for rooting, root number and root length.

3.6. Acclimatization

Sixty plantlets with well developed shoot and roots were taken out of the culture jars and washed with water to remove all traces of the medium. The plantlets were planted in cell tray for one month. Then, the plantlets were placed under plastic cover (as a shade) for two weeks and after another two weeks by removing the plastic cover. In first week of transfer, plantlets were watered morning and afternoon and after that watering interval was reduced. After one month plantlets were transfer into polythene bags (2:2:1 top forest soil, compost and sand respectively) and again covered by plastic cover for one week. In the first week of transfer, plantlets were watered frequently after that only two times per day (morning and evening).

3.7. Data Analysis

The collected data were analyzed using SAS (Version 9.2) software (SAS Institute Inc. 2008). The collected data were subjected to two and three way analysis of variance (ANOVA) according to the CRD model for factorial experiments and means were compared using Fisher's protected least significant difference test at the 5% significance level.

4. RESULTS AND DISCUSSION

4.1. The combined effect of BAP, TDZ and explant type on shoot multiplication

The analysis of variance indicated that the interaction effects of BAP, TDZ and explant type were highly significantly different (P<0.0001) for shoot number and shoot length. Moreover, there were highly significant differences (P=0.0012) and (P=0.0024) for leaf number and number of days to initial differentiation of shoots after sub-culturing, respectively (Appendix 1; Fig. 3).

Time taken for the shoot initiation was minimum (8.00±0.00 days) at 2 mg/l BAP combined with 0.3 mg/l TDZ, while it was maximum (10.67 days) and (11.33 days) in growth regulatory free medium for whole bud and section bud explants, respectively. The shoot initiation was early in TDZ supplemented media than that of BAP (Table1). Among the treatments, medium supplemented with 1 mg/l BAP in combination with 0.5 mg/l TDZ was the best combination of growth regulators for shoot number and shoot length from both explants. As shown in Table 1, shoot number showed differences in both explants with mean values of 5.33± 0.58 and 7.00±0.0 for whole and sectioned buds explants, respectively. The average length of shoots was 5.00±0.00 cm for whole buds and 4.767±0.58 cm for section buds. The lowest (2.33±0.58) shoot number was observed from the highest concentration of TDZ hormone and their highest combination followed by media with no hormones.

This reduction in number of shoots at concentrations beyond the stated levels could be due to the inhibitory effect of higher BAP and TDZ concentrations on the activities of endogenous auxins, which in turn is highly associated with apical dominance and bud dormancy (George *et al*., 2008). The current result is in agreement with the findings of Naz *et al. (2009)* who reported that the higher concentration of BAP decreases the shoot multiplication rate in turmeric among BAP concentrations (1-6 mg /l) tested and Rout *et al. (*1997) also reported that shoot multiplication decreases with increasing concentration of BAP level from 6 to 8 mg /l in ginger.

Table 1: The combined effect of BAP, TDZ and explants type on shoot multiplication

BAP (mg/l)	TDZ (mg/l)	Explants	Number of days	Shoot number	Shoot height	Leaf number*
0	0.0	W	$10.67^{b}\pm0.58$	$2.33^{k}\pm0.58$	$4.567^{bcd}\pm0.06$	$1.9^{bc}\pm0.00$
1	0.0	W	$10.00^{cd}\pm0.00$	$2.67^{jk}\pm0.58$	$4.833^{ab}\pm0.15$	$1.9^{bc}\pm0.00$
2	0.0	W	$9.33^{ef}\pm0.58$	$4.00^{fgh}\pm0.00$	$4.833^{ab}\pm0.15$	$1.97^{abc}\pm0.11$
3	0.0	W	$9.00^{fg}\pm0.00$	$4.33^{efg}\pm0.58$	$4.33^{def}\pm0.15$	$1.8^{cd}\pm0.17$
4	0.0	W	$10.00^{cd}\pm0.00$	$4.00^{fgh}\pm0.58$	$3.833^{ghij}\pm0.15$	$1.7^{de}\pm0.17$
0	0.1	W	$10.00^{cd}\pm0.00$	$3.67^{ghi}\pm0.58$	$4.567^{bcd}\pm0.06$	$1.8^{cd}\pm0.17$
1	0.1	W	$8.33^{ih}\pm0.58$	$3.33^{hij}\pm0.58$	$4.833^{ab}\pm0.15$	$1.47^{fg}\pm0.23$
2	0.1	W	$9.00^{fg}\pm0.00$	$4.00^{fgh}\pm0.00$	$4.33^{def}\pm0.15$	$1.6^{ef}\pm0.00$
3	0.1	W	$8.33^{hi}\pm0.58$	$5.00^{de}\pm0.00$	$4.00^{fgh}\pm0.00$	$1.47^{fg}\pm0.23$
4	0.1	W	$9.00^{fg}\pm0.00$	$2.67^{jk}\pm0.58$	$4.5^{bcde}\pm0.2$	$1.7^{de}\pm0.17$
0	0.3	W	$8.33^{hi}\pm0.58$	$3.33^{hij}\pm0.58$	$4.833^{ab}\pm0.15$	$1.6^{ef}\pm0.00$
1	0.3	W	$8.00^{i}\pm0.00$	$2.67^{jk}\pm0.58$	$4.00^{fgh}\pm0.00$	$1.2^{h}\pm0.23$
2	0.3	W	$8.00^{i}\pm0.00$	$2.67^{jk}\pm0.58$	$4.00^{fgh}\pm0.00$	$2.1^{a}\pm0.00$
3	0.3	W	$8.33^{ih}\pm0.58$	$3.33^{hij}\pm0.58$	$3.167^{mno}\pm0.15$	$2.1^{a}\pm0.00$
4	0.3	W	$8.33^{ih}\pm0.58$	$3.33^{hij}\pm0.58$	$3.067^{no}\pm0.12$	$1.7^{de}\pm0.17$
0	0.5	W	$8.00^{i}\pm0.00$	$5.33^{cd}\pm0.58$	$4.167^{efg}\pm0.35$	$1.6^{ef}\pm0.00$
1	0.5	W	$8.00^{i}\pm0.00$	$5.33^{cd}\pm0.58$	$5.00^{a}\pm0.5$	$1.6^{ef}\pm0.00$
2	0.5	W	$9.00^{fg}\pm0.00$	$5.00^{de}\pm0.00$	$3.33^{lmn}\pm0.15$	$1.6^{ef}\pm0.00$
3	0.5	W	$9.00^{fg}\pm0.00$	$4.67^{def}\pm0.58$	$3.73^{hijk}\pm0.25$	$1.7^{de}\pm0.17$
4	0.5	W	$10.00^{cd}\pm0.00$	$2.67^{k}\pm0.58$	$4.30^{def}\pm0.15$	$1.6^{ef}\pm0.00$
0	0.7	W	$9.00^{fg}\pm0.00$	$3.00^{ijk}\pm0.00$	$4.50^{bcde}\pm0.50$	$1.8^{cd}\pm0.17$
1	0.7	W	$9.00^{fg}\pm0.00$	$5.00^{de}\pm0.00$	$4.50^{bcde}\pm0.50$	$1.9^{bc}\pm0.00$
2	0.7	W	$9.00^{fg}\pm0.00$	$4.67^{def}\pm0.58$	$3.367^{klmn}\pm0.32$	$1.9^{bc}\pm0.00$
3	0.7	W	$10.00^{cd}\pm0.00$	$4.33^{efg}\pm0.58$	$3.83^{ghij}\pm0.16$	$1.6^{ef}\pm0.00$
4	0.7	W	$10.67^{b}\pm0.58$	$2.33^{k}\pm0.58$	$3.00^{no}\pm0.00$	$1.7^{de}\pm0.17$
0	0.0	S	$11.33^{a}\pm0.58$	$2.33^{k}\pm0.58$	$4.5^{bcde}\pm0.2$	$1.9^{bc}\pm0.00$
1	0.0	S	$10.00^{cd}\pm0.00$	$4.33^{efg}\pm0.58$	$4.4^{cde}\pm0.1$	$1.97^{abc}\pm0.11$
2	0.0	S	$9.00^{fg}\pm0.00$	$4.67^{def}\pm0.58$	$4.767^{abc}\pm0.21$	$2.03^{ab}\pm0.17$

Table 1: Continued

BAP (mg/l)	TDZ (mg/l)	Explants	Number of days	Shoot number	Shoot height	Leaf number
3	0.0	S	$8.67^{gh}\pm0.58$	$6.67^{ab}\pm0.58$	$4.4^{cde}\pm0.1$	$1.8^{cd}\pm0.17$
4	0.0	S	$10.00^{cd}\pm0.00$	$4.00^{fgh}\pm0.00$	$1.967^{r}\pm0.06$	$1.7^{de}\pm0.17$
0	0.1	S	$10.00^{cd}\pm0.00$	$4.33^{efg}\pm0.58$	$4.5^{bcde}\pm0.2$	$1.97^{abc}\pm0.11$
1	0.1	S	$9.67^{ed}\pm0.58$	$4.00^{fgh}\pm0.00$	$3.53^{ijklm}\pm0.06$	$1.8^{cd}\pm0.17$
2	0.1	S	$10.00^{cd}\pm0.00$	$2.67^{jk}\pm0.58$	$4.63^{abcd}\pm0.15$	$2.03^{ab}\pm0.17$
3	0.1	S	$8.33^{ih}\pm0.58$	$4.00^{fgh}\pm0.00$	$2.33^{qr}\pm0.06$	$1.6^{ef}\pm0.00$
4	0.1	S	$9.33^{ef}\pm0.58$	$6.00^{bc}\pm0.00$	$3.167^{mno}\pm0.12$	$1.7^{de}\pm0.17$
0	0.3	S	$9.67^{de}\pm0.58$	$3.00^{ijk}\pm0.00$	$4.4^{cde}\pm0.1$	$1.7^{de}\pm0.17$
1	0.3	S	$9.00^{fg}\pm0.00$	$6.67^{ab}\pm0.58$	$3.9^{ghi}\pm0.1$	$1.9^{bc}\pm0.00$
2	0.3	S	$8.00^{i}\pm0.00$	$5.33^{cd}\pm0.58$	$3.167^{mno}\pm0.12$	$2.1^{a}\pm0.00$
3	0.3	S	$9.00^{fg}\pm0.00$	$7.00^{a}\pm0.00$	$3.00^{no}\pm0.00$	$2.1^{a}\pm0.00$
4	0.3	S	$8.00^{i}\pm0.00$	$2.67^{jk}\pm0.58$	$3.167^{mno}\pm0.12$	$1.2^{h}\pm0.23$
0	0.5	S	$9.33^{ef}\pm0.58$	$7.00^{a}\pm0.00$	$4.63^{abcd}\pm0.15$	$1.9^{bc}\pm0.00$
1	0.5	S	$8.33^{hi}\pm0.58$	$7.00^{a}\pm0.00$	$4.767^{abc}\pm0.21$	$1.97^{abc}\pm0.11$
2	0.5	S	$8.33^{hi}\pm0.58$	$3.00^{ijk}\pm0.00$	$3.53^{ijklm}\pm0.6$	$1.97^{abc}\pm0.11$
3	0.5	S	$9.67^{de}\pm0.58$	$3.00^{ijk}\pm0.00$	$2.6^{pq}\pm0.00$	$1.9^{bc}\pm0.00$
4	0.5	S	$9.67^{de}\pm0.58$	$2.33^{k}\pm0.58$	$3.967^{fgh}\pm0.21$	$1.8^{cd}\pm0.17$
0	0.7	S	$9.33^{ef}\pm0.58$	$2.33^{k}\pm0.58$	$3.567^{ijkl}\pm0.15$	$1.9^{bc}\pm0.00$
1	0.7	S	$9.33^{ef}\pm0.58$	$2.67^{k}\pm0.58$	$2.833^{op}\pm0.15$	$1.8^{cd}\pm0.17$
2	0.7	S	$8.00^{i}\pm0.00$	$3.33^{hij}\pm0.58$	$4.767^{abc}\pm0.21$	$1.8^{cd}\pm0.17$
3	0.7	S	$10.33^{bc}\pm0.58$	$4.67^{def}\pm0.58$	$3.567^{ijkl}\pm0.15$	$1.6^{ef}\pm0.00$
4	0.7	S	$10.33^{bc}\pm0.58$	$2.33^{k}\pm0.58$	$2.33^{qr}\pm0.06$	$1.33^{gh}\pm0.23$
CV			**4.2**	**11.76**	**5.68**	**6.84**

Means within a column followed by the same letter are not significantly different using Fisher's protected least significant difference test at the 5% significance level.
Key,W=Whole bud,S=section bud,*= mean of transformed data.

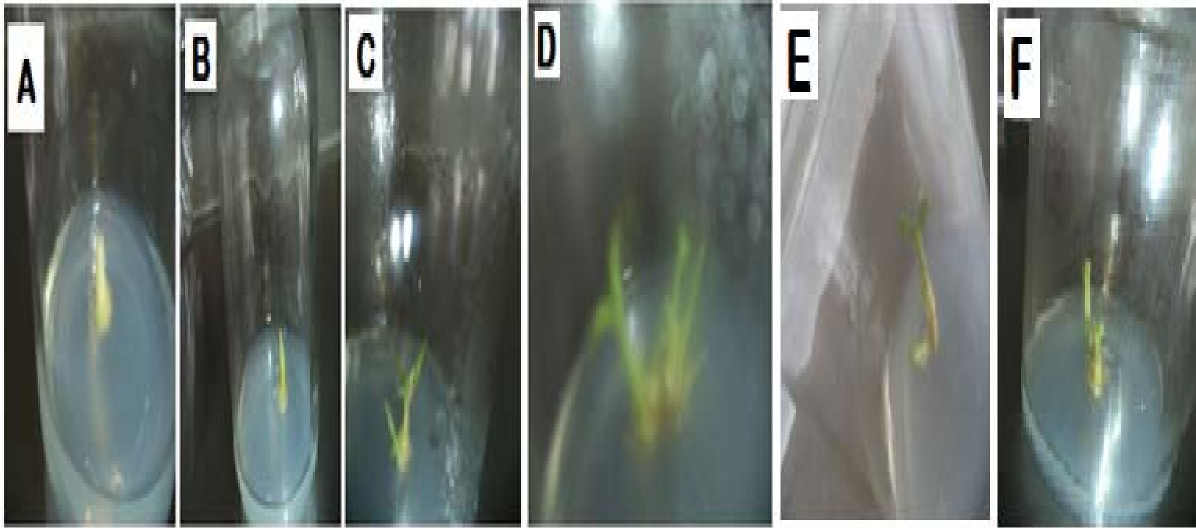

Figure1. The effect of BAP (2.0 mg/l) and TDZ (0.5mg/l) on shoot multiplication for whole buds; A) Explant during culturing, B) After 5th days 2mg/l BAP, C) After two weeks 2mg/l BAP,D) after four weeks 2mg/l BAP,E)After two weeks 0.5 mg/l TDZ, F) After three weeks 0.5 mg/l TDZ.

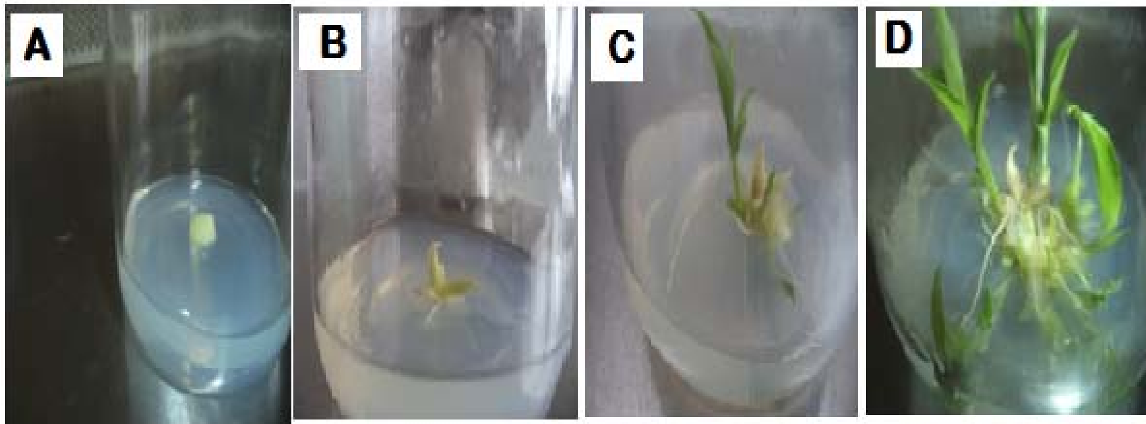

Figure2. The effect TDZ (0.5mg/l) on shoot multiplication for sectioned buds; A) Explant during culturing, B) After 5th days C,) After two weeks, D) After five weeks

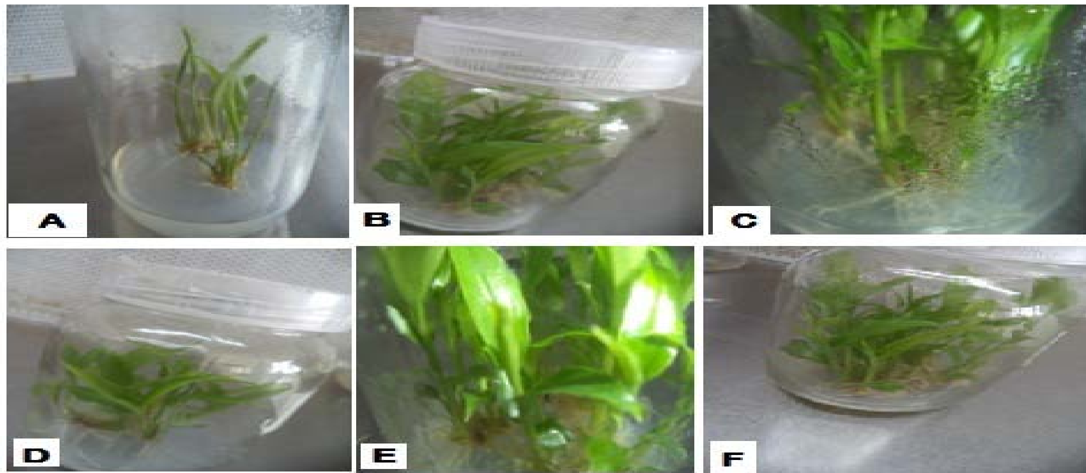

Figure3.)The Effect of Different Growth Regulators on Shoot Multiplication for whole buds and section bud explants(after four weeks).A) 4.0 mg/l BAP combined with 0.7 mg/l TDZ for whole bud, B) 1.0 mg/l BAP combined with 0.5mg/l TDZ for whole bud , C) 3.0 mg/l BAP combined with 0.3mg/l TDZ for whole bud. D) 4.0 mg/l BAP combined with 0.7 mg/l TDZ for section bud, E) 1.0 mg/l BAP combined with 0.5mg/l TDZ for section bud , F) 3.0 mg/l BAP combined with 0.3mg/l TDZ as bud section.

Increasing the concentration level of BAP from 0 to 2 mg /l has increased shoot number, shoot length and leaf number in both explants. Further increasing BAP from 3 mg/l to 4 mg/l decreased shoot number from 6.67±0.58 to 4.00±0.00, shoot length from 4.4±0.1 to 1.967±0.06 and leaf number from 1.8±0.58 to 1.7±0.58 for sectioned bud explants. Similarly, it showed a decrease from 4.33 to 4.00 for shoot length, from 4.33±0.58 to 3.83±0.58 for shoot number and from 1.8±0.58 to 1.7±0.58 for leaf number in the case of whole bud explants. This result is similar to that of Kambask and Santilata (2009) who reported BAP (2.0 mg /l) + NAA (0.5 mg /l) giving the best result for shoot multiplication (7.5 shoots per rhizome bud) whereas higher concentration of BAP (2.5-3.0 mg /l) with NAA (0.5 mg /l) exhibited callus formation with fewer number of shoots. But our result is contrary to that of Zuraida et *al. (2011*) who reported BAP at the highest dosage giving more stimulatory effect for shoot growth than the lowest dosage in the culture medium on ginger.

Increasing the concentration of BAP from 0 to 3.0 mg/l by keeping TDZ at 0.1mg/l showed a continuous decrease in the number of shoots (4.33±0.58 to 2.330.58),shoot length (4.5±.58to2.33±0.58) and leaf number (1.97±0.58to1.6±0.58) for section buds and shoot length (4.567 to 4.00) and leaf number (1.97±0.58to1.47±0.58) for whole bud explant. But increasing the concentration of BAP from 0 to 3.mg/l by keeping 0.3mg/l TDZ concentration showed a significant increase in shoot and leaf numbers from 3.00±0.00 to 7.00±0.00 and 1.7±0.58to2.1±0.58, respectively for section bud and from 2.33±0.58 to 3.33±0.58 and1.6±0.58 to2.1±0.58 for whole bud.

At this concentration, shoot length decreased from 4.4±0.1to 3.00±0.00 and from 4.83±0.15 to 3.167±0.12 cm for section buds and whole buds, respectively. In the current study, keeping the concentration of BAP at the same level, but varying TDZ concentration up to stated level increased shoot number. When the concentration of TDZ increased from 0 to 0.7 mg/l by keeping the concentration of BAP at 0 mg/l showed a decrease in shoot number from 4.33±0.58 to 2.67±0.58 and shoot length from 4.5 ±0.58 to 3.67±0.58 for sectioned buds and decreased shoot length from 4.567±0.58 to 4.5±0.58 and leaf number from1.7±0.17 to1.6±0.00 in whole buds. But, at 0.5 mg/l TDZ it increased to 4.63±0.58 for shoot length from sectioned buds and at 0.3mg/l TDZ it increased to 4.83 ±0.58 cm for shoot length from whole buds. However increasing concentration of TDZ from 0.0 mg/l to 0.5mg/l increased shoot length from 4.4±0.58 to 4.67±0.58, cm and shoot number from 2.33±0.58 to7.00±0.00 from section bud explants. For whole bud explant increase shoot length from 4.83±0.58 to 5.00±0.00 and shoot number from 2.67±0.58 to 5.33±0.58. This result in agreement with the finding of Ramage *et al*. (2002) and Hamirah *et al.(*2007). These authors reported that maximum number of (9.6 and 8.1) shoot per explants with MS medium supplemented with 0.5 mg/l TDZ respectively. The present result is different from that of Prathanturarug *et al*. (2003) who obtained 18.22 shoots/explants after 12 weeks, using 18.17µM TDZ rather than 0.5mg/l TDZ. In this study TDZ was more effective than BAP (figure 1 & 2)for shoot number which is similar to Carvalho et al. (2000) who stated that TDZ may be involved in the synthesis or accumulation of cytokinin in plant tissues which helps for multiple shoot induction.

In the current study, maximum shoot number with (7.00±0.00) was observed when bud section explants were cultured on MS supplemented 1mg/l BAP combined with 0.5 mg/l TDZ and at 0.5 mg/l TDZ only. This is similar to that of Hamirah *et al*.(2007) who stated that whole and sectioned-bud explants produced a mean of 4.6 and 5 shoots, respectively. However, by sectioning the bud explant into half, it helped to double the shoot number when compared with the whole bud.

This approach has been reported in pineapple micro propagation in which the shoot multi-plication rate has improved significantly De Almeida *et al*. (2002) who stated that longitudinal sectioning of the shoots used as explants proved to be a very good technique to improve the multiplication of shoots. It was found that the average number of shoots per explant (613.7) was high compared with only 69.1shoots/explant when the whole shoot was used as explant. Sectioning in meristematic regions can induce cell division due to the high mitotic ability of these cells (George, 1993). The stimulation of cell division, caused by the sectioning of the shoot, probably contributed for the differentiation of a higher number of adventitious buds and consequently a higher number of shoots produced per explant.

4.2. The Combined Effect of NAA and IBA on Rooting of micro shoots

The analysis of variance indicated that the effects of IBA and NAA were significantly different ($P<0.0001$) for number of days to rooting, root number and root length (Appendix 2). Time taken for root initiation was minimum (7.67±0.58 days) at 1.5 mg/l IBA combined with 0.5mg/l NAA, while it was maximum (10.67days) in growth regulator free medium and 0.5mg/l NAA only.

Table 2: The Combined Effect of NAA and IBA on Rooting of micro shoot

IBA(mg/l)	NAA(mg/l)	Number of days	Root number	Root length(cm)
0.0	0.0	10.67a±0.58	2.33^{k}±0.58	3.567^{d}±0.12
0.5	0.0	10.33^{ab}±0.58	3.00^{jk}±0.00	4.00^{bc}±0.00
1.0	0.0	10.00^{abc}±0.00	3.33^{ij}±0.58	4.00^{bc}±0.00
1.5	0.0	8.33^{fg}±0.58	8.67^{b}±0.58	4.33^{ab}±0.29
2.0	0.0	8.67^{ef}±0.58	4.00^{hi}±0.00	2.233^{g}±0.25
0.0	0.5	10.67^{a}±0.58	4.33^{gh}±0.58	3.567^{d}±0.12
0.5	0.5	8.33^{fg}±0.58	5.67^{ef}±0.58	4.00^{bc}±0.00
1.0	0.5	9.33^{cde}±0.58	6.67^{cd}±0.58	4.633^{a}±0.15
1.5	0.5	7.67^{g}±0.58	10.00^{a}±0.00	4.633^{a}±0.15
2.0	0.5	9.00^{cde}±0.00	1.33^{l}±0.58	2.067^{g}±0.12
0.0	1.0	10.00^{abc}±0.00	3.00^{jk}±0.00	3.00^{ef}±0.62
0.5	1.0	10.00^{abc}±0.00	5.33^{ef}±0.58	3.67^{cd}±0.29
1.0	1.0	9.33^{cde}±0.58	6.00^{de}±0.00	3.133^{e}±0.12
1.5	1.0	9.67^{bcd}±0.58	9.00^{b}±0.00	3.567^{d}±0.12
2.0	1.0	8.33^{fg}±0.58	6.00^{de}±0.00	4.00^{bc}±0.00
0.0	1.5	9.00^{cde}±0.00	7.00^{c}±0.00	3.133^{e}±0.12
0.5	1.5	8.33^{fg}±0.58	6.67^{cd}±0.58	4.33^{ab}±0.29
1.0	1.5	8.33^{fg}±0.58	6.00^{de}±0.00	3.67^{cd}±0.29
1.5	1.5	9.67^{bcd}±0.58	5.67^{ef}±0.58	3.167^{e}±0.29
2.0	1.5	8.67^{ef}±0.58	5.00^{fg}±0.00	2.067^{g}±0.12
0.0	2.0	9.33^{cde}±0.58	5.00^{fg}±0.00	2.067^{g}±0.12
0.5	2.0	8.67^{ef}±0.58	2.66^{jk}±0.58	3.133^{e}±0.12
1.0	2.0	9.00^{def}±0.00	2.66^{jk}±0.58	2.733^{f}±0.25
1.5	2.0	10.00^{abc}±0.00	3.33^{ij}±0.58	2.233^{g}±0.25
2.0	2.0	$9.00^{def\pm0.00}$	2.33^{k}±0.58	2.067^{g}±0.12
CV		**5.16**	**8.67**	**6.53**

Means within a column followed by the same letter are not significantly different using Fisher's protected least significant difference test at the 5% significance level.

A

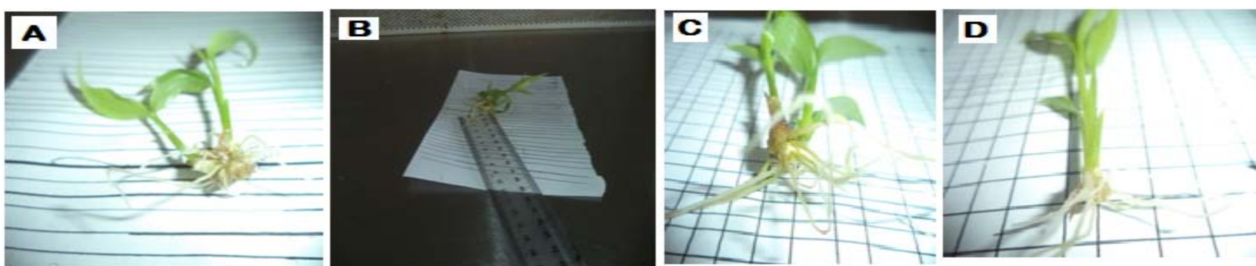

Figure 4: The Effect of Different Growth Regulators on rooting (after three weeks); A) PGRs free , B) 2 mg/l IBA combined with 2 mg/l NAA, C) 1.5mg/l IBA combined with 0.5mg/l NAA , D)1.5mg/l IBA combined with1mg/l NAA.

Both the highest mean root numbers per shoot (10.00±0.00) and mean root length (4.63±0.58 cm) were obtained when 1.5 mg/l IBA was combined with 0.5 mg/l NAA (Table 2 and figure4). The second highest mean root number (9.00±0.00) and root length (3.567±0.58) were also obtained at 1.5 mg/l IBA combined with 1mg/l NAA. Increasing IBA from 0.0 mg/l to 1.5mg/l by keeping NAA concentration at 0.0 mg/l showed a significant increase in the number of roots per shoot from 2.33±0.58 to 8.67±0.58 and mean root length from 3.567±0.58 to 4.33±0.58 cm. Increasing IBA concentration from 0.0 mg/l to 1.5mg/l by keeping NAA concentration at 0.5 mg/l showed a significant increase in the number of roots per shoot from 4.33±0.58 to 10.00±0.00 and mean root length from 3.567 ±0.58 to 4.633±0.58 cm. Increasing IBA from 0 mg/l to 2.0 mg/l by keeping NAA concentration at 1.5 mg/l showed a reduction in the mean root number per shoot and root length from 7.00±0.00 to 5.00±0.00 and (3.133±0.58 to 2.067±0.58)cm, respectively. Increasing IBA from 0.0 mg/l to 1.5mg/l by keeping NAA concentration at 1.0 mg/l showed a significant increase in the number of roots per shoot from 3.00 to 9.00 and mean root length from 3.00±0.00 to 3.567±0.58 cm .

In all higher concentration and combinations of the two auxins, lower root number and root length were recorded. The combination of 2.0 mg/l concentration each of the two auxins (the highest concentration) gave 2.33±0.58 and 2.06±0.58 mean root number and mean root length (cm), respectively. When concentration of IBA increased from 0mg/l to 1.5 mg/l, it resulted in an increase of both number of roots per shoot and mean root length. However, the combination of 2.0 mg/l IBA and 1.5 mg/l NAA resulted in 5.00±0.00 and 2.06±0.58 mean root number and mean root length, respectively, which indicates the inhibitor effect of high concentration of auxins. Dipti *et al.* (2005) reported maximum rooting on a medium

supplemented with 0.5 mg/l NAA turmeric. Meenakshi *et al.* (2001) also reported the highest rooting on a medium supplemented with 0.3 mg/l NAA in micro propagation of turmeric. In the present study, contrary to Dipti *et al.* (2005) and Meenakshi *et al.* (2001) the maximum rooting to multiple shoots were observed on MS medium with 1.5mg/l IBA combined with 0.5 mg/l NAA. In this study, IBA was more effective compared to NAA which is similar to the results of Bhagyalakshmi and Singh (1988) who reported that IBA was more effective than NAA for root formation in meristem culture. Contrary to the present study, Kambaska and Santilata (2009) stated NAA was more effective than IBA in root induction. According to Sit and Tiwari (1998) rooting was best on 0.5 mg /l IBA but rooting of micro rhizomes did not take place in the absence of IBA which is contrary to the present result. In present study, even if there were rooting of shoots on MS basal medium, the root numbers recorded were the lowest (2.33±0.58) in the absence of IBA and the best was on 1.5mg/l IBA combined with 0.5 mg /NAA.

The number and length of roots increased with the increased concentration of NAA and IBA applied. However, when the concentration of both growth regulators was 2 mg/l, both root number and root length were decreased. In agreement with the present observation, George and Sherrington (1984) reported a decrease of root number when the concentration of IBA was greater than 2.0 mg/l. They indicated the inhibitory effect of high concentration of auxins on root formation of plants as a cause for such decrease. Weiler (1984) also reported the inhibition of root elongation by higher concentrations of growth regulators and stated ethylene deposition as the reason.

4.3. Acclimatization

Among the acclimatized plantlets, 80% survived (Fig 5), which is similar to the results of Samsudeen *et al.* (2004) that 85 per cent success when plants were transplanted in potting mixture of garden soil, sand and vermiculite in equal proportions and kept in humid chamber initially for 22 to 30 days. This result is in contrast with Ali *et al.* (2004) and Salvi *et al.* (2000) who had 100% and 95% who successfully transferred turmeric plantlets to the greenhouse in pots containing soil with equal amount of sand + clay + compost and plants of turmeric were transferred to sterilized soil in paper cups for 3 to 4 weeks .

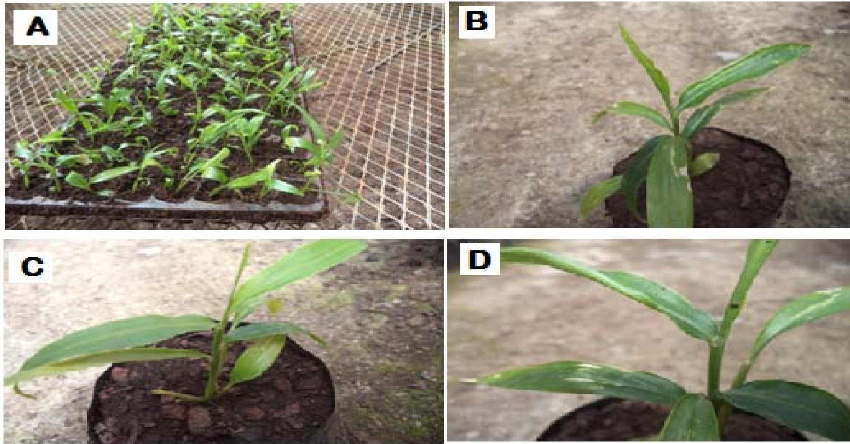

Figure 5: Acclimatization; A) During transplanting in cell tray, B) During transplanting in polythene tube, C) After three weeks, D) After six weeks

5. CONCLUSION AND RECOMENDATION

Ginger is the second most widely cultivated spice in Ethiopia, next to chilies. However, its expansion is limited by different factors such as inefficient propagation method and disease transmission, which is a hindrance to get enough and clean planting material.

Therefore, this study was initiated to develop a protocol for *in vitro* propagation of ginger. Rhizome buds were used to establish sprouts which were used as source of explant. After the rhizome buds sprouted, they were excised and sterilized by 70% ethanol for 5 min followed by 50% local bleach (berekina) for 30 min. The sprouted rhizome buds were cultured as whole and longitudinally sectioned into half in MS medium supplemented with different concentrations of BAP and TDZ with antibiotics (streptomycin and gentamicine 1mg/l and tetracycline 15mg/l) to see their effect on shoot multiplication. Multiple clums of shoots were separated as individual shoot and well developed shoots were transferred into 1/2MS medium supplemented with different combination of NAA and IBA for root induction. The experiments were carried out in completely randomized design (CRD) with three replications per treatment and three explants per jar. The cultures were kept at a temperature of 25 $\pm 2^{o}$C and light intensity of 2,000-µmolM^2S^2 produced from cool white fluorescent tubes for 16 h photoperiod. Data like number of days for shoot initiation after subculture, shoot number, shoot length and leaf number, for shoot multiplication and number of days for root initiation, root number and root length were collected, and analyzed using SAS software.

The analysis of variance revealed that the effect of the growth regulators were highly significant ($P<0.0001$) for both shoot and root parameters, i.e., shoot number, shoot length, days to root initiation, root number and root length. Both maximum shoot number and shoot length was recorded at 1mg/l BAP combined with 0.5mg/l TDZ and the maximum leaf number observed at 2 and 3mg/l BAP combined with 0.3mg/l TDZ. The effect of BAP was more than that of TDZ for shoot length in both explants but effect of TDZ was than that of BAP in time taken for shoot initiation, shoot number and leaf number in both explants. Whole bud explant was better than sectioned rhizome buds in case of shoot length. However, time taken for shoot initiation, shoot number and leaf number were more in sectioned buds. BAP at

a concentration of 1.0mg/l combined with 0.5mg/l TDZ resulted in early initiation (8.00±0.00) for whole bud explant. Maximum mean shoot number per explant (7.00±0.00), mean shoot length (5.00±0.00) cm and the maximum mean leaf number (2.1±0.00) were recorded at BAP 2 and 3mg/l combined with 0.3mg/l TDZ.

When the concentration of IBA was greater than that of NAA, relatively better rooting results were recorded. The minimum (7.67±0.58 days), highest mean root numbers per shoot (10.00±0.00) and mean root length (4.633±0.15 cm) were obtained at 1.5 mg/l IBA combined with 0.5 mg/l NAA.

In this study, BAP at a concentration of 1.0 mg/l combined with 0.5mg/l TDZ was found to be optimal concentration for shoot multiplication. Efficient *in vitro* rooting of individual shoot culture was obtained at 1.5 mg/l IBA combined with 0.5 mg/l NAA which resulted in both the highest number of roots per shoot (10.0±0.00) and mean root length (4.633±0.15) cm.

Hence, MS medium supplemented with 1.0 mg/l BAP combined with 0.5mg/l TDZ could be recommended for shoot multiplication and sectioned rhizome bud explants on 1/2MS medium supplemented with 1.5mg/l IBA combined with 0.5mg/l NAA could be recommended for *in vitro* rooting of ginger from rhizome bud explants. However, further optimization of this protocol using more genotypes may be required for mass propagation of ginger as this study is limited to a single cultivar.

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7. Appendices

Appendix Table1: ANOVA summary of the effects of hormone and explant on varied growth parameters of ginger shoot

		Shoot length		**Shoot number**		**leaf number**		**Number of days**	
Source of variation	DF	Mean Square	Pr > F	Mean Square	Pr > F	Mean Square	Pr > F	Mean Square	Pr >F
BAP	4	7.32156667	<.0001***	6.4600000	<.0001***	4.33333333	<.0001***	3.56000000	<.0001***
TDZ	4	1.75023333	<.0001 ***	3.0600000	<.0001***	1.35000000	<.0001***	7.57666667	<.0001***
BAP*TDZ	16	1.35477500	<.0001***	7.1058333	<.0001***	1.93333333	<.0001 ** *	2.53916667	<.0001***
Explants	1	5.49126667	<.0001***	8.1666667	<.0001***	4.86000000	<.0001 ** *	1.30666667	0.0043**
BAP*Explants	4	1.09976667	<.0001***	2.0333333	<.0001***	1.56000000	<.0001** *	1.24000000	<.0001***
TDZ*Explants	4	0.19210000	0.0055**	8.5000000	<.0001***	1.91000000	<.0001***	0.42333333	0.0317*
BAP*TDZ*Explants	16	0.90205833	<.0001***	4.8041667	<.0001***	0.44333333	0.0024**	0.41916667	0.0012**

DF= Degree of freedom, Explants= explant type, *= Significant **= highly significant, ***= Very highly significant.

AppendixTable2: ANOVA summary of the effects of hormone and explant on varied growth parameters of ginger roots *in vitro*

	Root height	Root number	Number of days
Source of			

Variation	DF	Mean Square	Pr > F	Mean Square	Pr > F	Mean Square	Pr > F
IBA	4	4.44233333	<.0001***	28.5333333	<.0001***	2.84666667	<.0001** *
NAA	4	4.10266667	<.0001** *	22.6000000	<.0001** *	1.18000000	0.0014**
IBA*NAA	16	1.05041667	<.0001** *	10.0083333	<.0001***	1.94666667	<.0001***

DF= Degree of freedom, Explants= explant type, *****= Significant **= highly significant, ***= Very highly significant.

Appendix Table 3: Composition of MS media used for all experiments

Category	Chemicals	Conc. in medium (mg/l)
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
Micronutrients	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA2H_2O$	33.6
	KI	0.83
	H_3BO_4	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.H_2O$	0.25
	$CuSO_4.5\ H_2O$	0.025
	$CoCl_2.6\ H_2O$	0.025
Organic supplements	Myoinositol	100
	Nicotinic acid	0.05
	Pyridoxine HCl	0.05
	Thiamine HCl	0.05
	Glycine	0.02
Carbon source	Sucrose	30,000
Gelling agent	Agar	8000
Growth regulators	BAP	100/100ml
	TDZ	100/100ml
	IBA	100/100ml
	NAA	100/100ml1